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EVALUATION, VALIDATION, AND DEMONSTRATION OF A TOTAL PROTEIN ASSAY FOR APPLICATION TO BIOTOXIN FATE STUDIES

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14. ABSTRACT
This report summarizes efforts to evaluate, optimize, and validate a commercially available analytical assay for the quantitative determination of total protein in aqueous sample matrices. The data generated during this study will be used to support on-going investigations into the fate of bio-toxins in aqueous environments. The optimized assay was found to be repeatable, reproducible, and not subject to interference by several different water matrices. The method limit of detection was determined to be 0.350 mg/L, and the method limit of quantization was determined to be 1.17 mg/L. The linear range was determined to be 1.00 to 25.0 mg/L of total protein.

15. SUBJECT TERMS

Total Protein	Ricin	SEB	BSA
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EXECUTIVE SUMMARY

The findings of this study support simulation of battlespace environment, environmental surveillance, decontamination, and individual protection in general. Biological Agent Fate and the Joint Services Agent Water Monitor (JSAWM) are specific programs supported by this study.

In support of the primary focus of this effort, an analytical assay for the quantitative determination of total protein was evaluated, optimized, and validated. The first total protein assay evaluated was the Micro BCA assay (Pierce Chemical Company, Rockford, IL). The Micro BCA assay requires a sample incubation time of 2 hr to improve upon several aspects of the total protein assay, the decision was made to discontinue efforts using the Micro BCA assay and begin evaluation of the Coomassie® Plus Protein Assay (Pierce Chemical Company). This second assay is better adapted to the microplate reader and requires smaller sample volumes, while maintaining the same or slightly better detection limit. Additionally, fewer preparation steps are involved than with the Micro BCA assay, and the sample incubation period is only 10 min.

Using the Coomassie® Plus Protein Assay, the individual protein response for bovine serum albumin (BSA), staphylococcal enterotoxin B (SEB), and lysozyme in the desired concentration range of 1 to 25 mg/L was not statistically different among the three proteins. The sensitivity of ricin response was, on average, 47% of the sensitivity of BSA, SEB, and lysozyme. Reproducibility and repeatability experiments with BSA demonstrated that the assay provides reproducible and consistent results. Four water matrices relevant to biotoxin fate (deionized water, synthetic tap water, distilled water with residual chlorine, and phosphate buffered saline solution) were evaluated for potential interference with this assay. Water with residual chlorine was the only sample matrix determined to interfere with the assay. This interference occurred at the high end of the linear concentration range.

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PREFACE

The work described in this report was authorized under Contract No. DAAD13-03-D-0017. This work was started in April 2005 and completed in November 2006. The primary purpose of this work was to evaluate, optimize, and validate an analytical assay for the determination of total protein in aqueous sample matrices.

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1. INTRODUCTION

The study of protein toxin fate requires the means to assess the hazard specific to the biological warfare (BW) agent of interest. Hazard assessment for BW agents has relied upon data from their detection and identification. However, these technologies can suffer from the potential for false positives (e.g., identification of protein toxins typically relies on their interaction with antibodies). A non-toxic fragment of the protein toxin that binds to the antibody would trigger a false positive. Thus, although simple detection and identification of a sample containing protein toxin offers a preliminary hazard assessment, they do not provide for a detailed biological toxin fate study to obtain information for battlespace modeling. The toxic or hazardous nature of a protein toxin is associated with three molecular-level properties: mass, biochemical activity, and molecular fold. For Biological Toxin Fate studies to obtain meaningful persistence data, the program must consider all three of these properties (mass, fold, and activity) for a correct hazard assessment. Mass characterization includes determination of molecular mass, which will be affected by any chemical reaction that alters the protein's molecular structure, and total mass or concentration of protein. The effort reported here focused on the optimization and validation of an analytical assay for determining one of these properties, namely concentration of total protein. Preliminary results on this effort were previously reported at the 2005 Scientific Conference on Chemical and Biological Defense Research.[1]

1.1 Background.

Although many assays exist to determine total protein content in a variety of sample matrices, there are seven basic approaches to determining total protein.[2] The first approach is the Lowry assay, which is based on formation of protein-copper complexes and subsequent reduction with Folin-Ciocalteu Reagent.[3] The second approach is acidic hydrolysis of the protein, followed by detection of the amino acid residues with ninhydrin reagent.[4] The third approach is the biuret assay, which relies on the chelation of cupric ion by the protein.[5] The fourth approach involves measuring the ultraviolet (UV) absorbance, and then using the extinction coefficient to calculate protein concentration.[6] The fifth approach is the bicinchoninic acid (BCA) assay, which relies on the reduction of cupric ion to cuprous ion by the protein.[7] The cuprous ion is then detected using a reagent containing bicinchoninic acid. The sixth approach is the Bradford assay, which relies on the binding of Coomassie brilliant blue G-250 dye (CBBG) to the protein.[8] This binding occurs at the arginine, tryptophan, tyrosine, histidine, and phenylalanine residues of the protein. The seventh approach is gravimetric, which is not often used in current practice and relies on obtaining a weight of the dried protein solution.[9] Characteristics of these assays are summarized in Table 1.

The characteristics summarized in Table 1 were used for the initial down selection process, to decide which commercial assays to evaluate during this effort. Based on the reported characteristics, the Lowry and biuret assays were dropped from consideration due to the relatively large sample volume required and/or high detection levels. The ninhydrin assay was not considered due to large sample volume and long analysis time. The UV absorbance assay, while not directly evaluated during this effort, was already being implemented as part of the Biological Toxin Fate program, and was not considered during this effort. The gravimetric assay was dropped from consideration due to the long analysis time and high detection levels. The BCA and Bradford dye binding assays had relatively short analysis times and detection limits consistent with the objectives of the biological toxin fate program. These two assays were selected for evaluation during this effort.

Table 1. Characteristics of Assays Used to Determine Total Protein[2]

Assay Description	Typical Sample Size Required (mL)	Typical Analysis Time (min)[a]	Typical Detection Range (mg/L)
Lowry	1.00	60	100 – 600
Ninhydrin	1.00	180[b]	20 – 50
Biuret	1.00	60	1,000 – 10,000
UV Absorbance	1.00	30	30 – 300
BCA	0.10	60	200 – 1,000
Bradford	0.10 – 0.20	30	60 – 300
Gravimetric	0.50 – 10.0	30[b]	2,000 – 10,000

a. Includes sample incubation time.
b. Indicated time is spread over two days.

1.2 Study Objectives.

The primary goal of this study was to adapt a commercially available assay for determining total protein concentration in aqueous matrices under conditions relevant to an on-going toxin fate program.[10] In addition, once adapted, the assay was to be validated for suitability to application in the toxin fate experiments. For a total protein assay to be suitable for application to the toxin fate program, the following conditions should be met:

- Sample preparation should be minimal, and total analysis time should be less than 30 min.
- The assay should be adaptable to execution in a plate reader format for high sample throughput.
- The assay should be applicable to water matrices used in toxin fate studies.
- The assay should detect a variety of protein toxins.
- The assay should have adequate linear range and low detection limits.

- The assay must be accurate, reproducible, and repeatable.

2. EXPERIMENTAL PROCEDURES

This section describes the experimental procedures and analytical methods used during this project. Analyses performed using standard methods and methods published in the open literature are referenced in the results section of this report.

2.1 Microplate Reader.

There are several well-documented protein assays of varying degrees of complexity already established.[2-9] One of the underlying goals of this project was to choose an assay that was quick and convenient, and at the same time required a minimal amount of hazardous chemicals and toxic proteins. The approach of combining a spectrophotometric microplate reader with a colorimetric protein assay was used to accomplish this goal. The use of a microplate reader for analysis limits the combined volume of the sample and reagent to a maximum of 300 μL per well when using a standard plate, with detection accomplished at a single wavelength. An endpoint absorbance reading can be recorded in < 15 min. Depending upon the particular assay chosen, sample preparation may be carried out directly in the individual wells of the microplate by the combination of sample with the commercially prepared assay reagents. The microplate reader used in the optimization and validation of this assay was the VersaMax™, available from Molecular Devices (Sunnyvale, California).

2.2 Standards.

The protein standards used were obtained from commercial sources and of the highest purity available. The bovine serum albumin, used as the standard, was purchased from Pierce, Rockford, IL at a concentration of 2.0 mg/mL in 0.9% NaCl solution containing sodium azide. A second source of bovine serum albumin was also used in some experiments, and was purchased from Sigma-Aldrich, Milwaukee, WI at a concentration of 1.0 mg/mL in 0.15 M NaCl solution containing sodium azide. Ricin was purchased from Vector Laboratories, Burlingame, CA at 5.0 mg/mL in 10 mM phosphate, 0.15 M NaCl, 0.08% sodium azide solution, pH 7.8. Lysozyme from chicken egg white was purchased from Sigma-Aldrich in lyophilized powder form, protein content approximately 95%. Lysozyme stock standards were prepared in the laboratory by dilution using Class A volumetric glassware. SEB was purchased from Sigma-Aldrich (Milwaukee, WI) in a lyophilized powder form, protein content approximately 0.25 wt %. SEB stock standards were prepared in the laboratory by dilution using Class A volumetric glassware.

2.3 Reagents.

All chemicals utilized were typically of ACS reagent grade, and were used as received from the manufacturer. All neat reagents were stored in a desicator. For the assay, the following chemicals were used: 1) from Sigma-Aldrich (Milwaukee, WI) - potassium dihydrogenphosphate (KH_2PO_4, CAS No. 7778-77-0), dibasic potassium phosphate (K_2HPO_4, CAS No. 7758-11-4), sodium chloride (NaCl, CAS No. 7647-14-5), 1 N sodium hydroxide solution (NaOH, CAS No. 1310-73-2), 1N hydrochloric acid (HCl, CAS No. 7647-01-0), and pH buffer solutions; 2) from Pierce Chemical Company (Rockford, IL) - Coomassie® Plus Protein Assay reagent.

For preparation of water matrices, chemicals of specific purity levels were employed. Sodium hydrogen carbonate ($NaHCO_3$, 99.7+%, CAS No. 144-55-8), magnesium sulfate hepahydrate ($MgSO_4 \bullet 7H_2O$, 98+%, CAS No. 10034-99-8), potassium hydrogen phosphate (K_2HPO_4, 98+%, CAS No. 7758-11-4), potassium dihydrogenphosphate (KH_2PO_4, 99+%, CAS No. 7778-77-0), ammonium sulfate [$(NH_4)_2SO_4$, 99+%, CAS No. 7783-20-2], sodium chloride (NaCl, 99+%, CAS No. 7647-14-5), iron (II) sulfate hepahydrate ($FeSO_4 \bullet 7H_2O$, 98+%, CAS No. 7782-63-0), sodium nitrate ($NaNO_3$, 99+%, CAS No. 7631-99-4), calcium sulfate ($CaSO_4$, 99%, CAS No. 7778-18-9), sodium hydroxide (NaOH, 97+%, CAS No. 1310-73-2), and hydrochloric acid (HCl, 37% in water, CAS No. 7647-01-0) were purchased from Sigma-Aldrich (Milwaukee, WI). The Suwannee River Fulvic Acid standard (Cat No. 1S101F) was purchased from the International Humic Substances Society (St. Paul, MN) The calcium hypochlorite [$Ca(OCl)_2$, CAS No. 7778-54-3)] was provided by the U.S. Army Edgewood Chemical Biological Center (APG, MD).

The ASTM Type I water was obtained from an in-house system (18 MS, Nanopure, Barnstead, Dubuque, IA). The ASTM Type III water was obtained from an in-house electric still (Corning MP-6A, Mega-Pure).

2.4 Aqueous Test Matrices.

There were three different water matrices, in addition to the baseline phosphate buffered saline (PBS, 10 mM PO_4^{-2}, 0.15 M NaCl, pH = 7.8), evaluated during this effort. The three water matrices are also used in the JSAWM Program* and were selected to represent a baseline case, potential source water for fielded troops, and a finished drinking water for fielded troops. These three matrices are, respectively, ASTM Type I deionized water (DIW), "synthetic" tap water (STW), and ASTM Type III water with 1-3 mg/L free residual chlorine (CLW). A representative batch of each water was characterized during a previous study,[11] and the results are summarized in Section 2.4.2.

*Morrissey, K.M.; Connell, T.R.; Crosier, R.B.; Sommerville, D.R.; Jensen, J.L.; and Durst, H.D. *Evaluation of Enzyme-Based Tickets for Detection of Cholinesterase Inhibitors in Water: Application to the JSAWM Program*; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, unpublished data 2007.

2.4.1 Preparation of Aqueous Test Matrices.

The DIW representing the baseline JSAWM water matrix was generated in-house using an on-demand system. A new batch of DIW was prepared each day of testing, and was stored in an acid-cleaned polycarbonate carboy.

The STW representing the JSAWM source water matrix utilized in this study was prepared, with one deviation, in accordance with an established recipe.* The deviation involved adding a double aliquot of fulvic acid stock solution, instead of fulvic and humic stock solutions. This deviation was not considered significant, as the total organic concentration in the test matrix was maintained. The primary stock solutions were prepared with ASTM Type I deionized water, and were stored in opaque HDPE bottles at 4 °C. The primary stock solutions were presumed to have a shelf-life of 6 months, though no stability studies were performed. The STW (example for 1 L) was prepared by adding approximately 500 mL of ASTM Type I deioinzed water to a 1-L Class A volumetric flask, then adding the primary stock solutions described in Table 2. The order of addition was not systematically evaluated, but was always in the order listed in Table 2. The volume was then brought to the mark with ASTM Type I de-ionized water, and the flask inverted to mix the contents. After waiting 15-20 min, the pH was determined. The pH should be between 7.6 and 7.8. If an adjustment was required, 1% HCl or NaOH was used to adjust the pH. The prepared STW was stored in an acid cleaned polycarbonate carboy at 4°C, and had a shelf life of seven days.

Table 2. Preparation of 1 L of Synthetic Tap Water

Chemical Formula	Concentration of Primary Stock (mg/L)	Primary Stock Added to One Liter (mL)	Final Concentration (mg/L)
$NaHCO_3$	10,000	10.0	100
$MgSO_4 \bullet 7H_2O$	1,000	13.4	13.4
K_2HPO_4	1,000	0.7	0.700
KH_2PO_4	1,000	0.3	0.300
$(NH_4)_2SO_4$	100	0.1	0.0100
NaCl	100	0.1	0.0100
$FeSO_4 \bullet 7H_2O$	10.0	0.1	0.001
$NaNO_3$	1,000	1.0	1.00
$CaSO_4$	1,000	27.0	27.0
Fulvic Acid*	1,000	2.0	2.00

*IHSS Suwannee River Fulvic Acid Standard

*Morrissey, K.M.; Connell, T.R.; Crosier, R.B.; Sommerville, D.R.; Jensen, J.L.; and Durst, H.D. *Evaluation of Enzyme-Based Tickets for Detection of Cholinesterase Inhibitors in Water: Application to the JSAWM Program*; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, unpublished data 2007.

The CLW representing the JSAWM finished water matrix, was prepared in a two-step process. The first step was to prepare a stock solution of calcium hypochlorite by adding 20 mL of ASTM Type I deionized water to 1.00 ±0.05 g of HTH in a 22 mL glass vial. This mixture was then vigorously mixed for 1-2 min. After letting the undissolved solids settle for 5-10 min, 15 µL of the HTH supernatant was added to 100 mL of ASTM Type III water in a 125 mL amber glass bottle. The free residual chlorine should be between 1 and 3 mg/L, and was measured with a test strip (HF Scientific, Free Chlorine Micro-Check). The HTH stock solution is not stable, and was prepared just prior to preparation of each batch of CLW. The CLW was prepared each day of testing, was stored in an amber glass bottle, and was periodically tested throughout the day to ensure chlorine levels stayed between 1 and 3 mg/L free residual chlorine. The amber bottle was conditioned with the chlorinated water prior to the first use by allowing it to soak overnight. In addition, the old chlorinated water was left in the bottle until a new batch was made. This reduced the chlorine demand of the bottle surfaces.

2.4.2 Characterization of Aqueous Test Matrices.

During another study,* a representative batch of each test matrix was prepared using the procedures described in Section 2.4.2 and chemically characterized. The test matrices were analyzed in accordance with standard Environmental Protection Agency (EPA) methods. Sample pH was determined using EPA Method 150.1,[11] specific conductance was determined using EPA Method 120.1,[12] acidities were determined using EPA Method 305.2,[13] alkalinities were determined using EPA Method 310.1,[14] total hardness was determined using a modification of EPA Method 130.2,[15] total dissolved solids were determined using EPA Method 160.1,[16] total suspended solids were determined using EPA Method 160.2,[17] total organic carbon was determined using EPA Method 415.1,[18] and total chlorine residual was determined using EPA Method 330.4.[19]

The results are summarized in Table 3. The concurrently run QC samples, such as laboratory controls and sample matrix spikes, were all within acceptable quality limits. There was only one anomaly during the analysis of these samples. The EPA Method 150.1 used to determine pH requires the sample be analyzed immediately. The samples were held for 2 days before the pH values were determined. The samples were stored at 4 °C, and the deviation is not believed to have had a significant impact on the results.

2.5 Selection of Commercial Total Protein Assay.

In the initial stages of this effort, the Micro BCA Protein Assay kit (Pierce Chemical Company, Cat. No. 23235) was evaluated. This kit utilized bovine serum albumin as the reference standard protein and reported a linear working range of 0.5-20 mg/L.[7] This assay was noted for less protein-to-protein variation than dye binding assays.[7] Performing the assay

*Morrissey, K.M.; Connell, T.R.; Crosier, R.B.; Sommerville, D.R.; Jensen, J.L.; and Durst, H.D. *Evaluation of Enzyme-Based Tickets for Detection of Cholinesterase Inhibitors in Water: Application to the JSAWM Program*; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, unpublished data 2007.

involved the preparation of a working BCA reagent from three individual stock solutions supplied in the kit. Calibration solutions were prepared by serial dilution of the bovine serum albumin in DIW. The working reagent was combined with the standard solution in a 1:1 ratio and the resulting solution was incubated at 37 °C for 2 hr. Once the incubation period was completed, the absorbance at 562 nm was measured. The assay was designed for use with cuvettes in a spectrophotometer, however it was reported to be adaptable for use in the microplate reader.[7]

Table 3. Chemical Characteristics of Representative Batches of Aqueous Test Matrices

Chemical Parameter	Aqueous Test Matrix		
	DIW	STW	CLW
pH[a]	6.1	7.9	8.2
Specific Conductance (μS/cm)	3.7	172	12.3
Acidity (to pH 3.7; mg/L)	ND[b]	ND[b]	ND[b]
Acidity (to pH 8.3; mg/L)	0.43	ND[b]	1.0
Alkalinity (to pH 4.5; mg/L)	0.44	58.8	3.5
Alkalinity (to pH 8.3; mg/L)	ND[b]	ND[b]	ND[b]
Total Hardness (mg/L)	ND[b]	25.7	5.4
Total Dissolved Solids (mg/L)	ND[b]	100	11.5
Total Suspended Solids (mg/L)	ND[b]	ND[b]	ND[b]
Total Organic Carbon (mg/L)	ND[b]	1.1	ND[b]
Total Chlorine Residual (DFP, mg/L)[c]	NA[d]	NA[d]	4.8

a. pH determined 2 days after the samples were prepared.
b. Analyte was not detected.
c. This is total chlorine residual, not free residual chlorine.
d. Not determined in this sample matrix.

The adaptation of this assay to the microplate reader raised the reported lower end of the linear working range to 2 mg/L, with a reported linear range of 2-40 mg/L.[7] In this effort a calibration study using BSA was performed in the range of 1-40 mg/L. While a linear response was demonstrated over this range, the need for an assay with a similar detection limit with a shorter sample incubation time was preferred. An assay requiring a 2-hr incubation time would not allow the sample throughput required during some biotoxin fate studies.

To improve upon several technical and logistical aspects of the total protein assay, a decision was made to discontinue evaluation of the Micro BCA assay and begin evaluation of the Coomassie® Plus Protein Assay (Pierce Chemical Company, Cat. No. 23236). This assay was adaptable to the microplate reader and required smaller sample volumes than the Micro BCA assay. The method detection limit was reported to be similar to the Micro BCA assay.[8] In addition, fewer preparation steps are involved than with the Micro BCA assay and the sample incubation period was only 10 min, compared to 2 hr for the Micro BCA assay.

2.6 Assay Optimization and Validation.

This section describes the experiments conducted during the evaluation, optimization, and validation of an assay for the determination of total protein in aqueous matrices. This assay is based on the commercially available Coomassie® Plus Protein Assay.[8]

An area of concern regarding protein assays was temperature regulation of the laboratory environment. The vendor reports that the absorbance measurements obtained at 595 nm with this assay are dependent upon the temperature of the reagent to some extent and that the reagent should remain at a constant temperature (i.e., room temperature) during the assay.[8] Ambient temperature of laboratories can fluctuate significantly throughout the day, and vary greatly from day to day. To eliminate this variable, the incubation temperature was maintained at a constant 37 °C, ensuring that all endpoint measurements were taken at a uniform temperature.

2.6.1 Reaction Time.

The reaction product is not stable, and the vendor recommended a consistent incubation time of 10 min prior to obtaining an absorbance reading.[8] The absorbance readings are also somewhat dependent on temperature,[8] and the reactions are typically run at room temperature (i.e., 25 °C). Because this assay was run at 37 °C due to equipment limitations and the vendor conditions were based on standards prepared in phosphate buffer, experiments to evaluate reaction time were performed.

The appropriate reaction time was determined by preparing standard solutions of BSA in PBS, DIW, STW, and CLW. The BSA concentration was 12.5 mg/L, and was chosen because it was the mid-point of the anticipated linear range. The solutions were prepared as 3 independent replicates, and analysis was started immediately after preparation. A total of 16 wells were analyzed for each replicate, with eight wells of blank matrix analyzed. An average of 36 time points was collected for each plate, over the course of 50 min. The resulting absorbance values were corrected for the background absorbance of the blank matrix.

2.6.2 Individual Protein Response.

The vendor of the Coomassie® Plus assay recommended that, for greatest accuracy, the calibration curve be prepared from a pure sample of the target protein. However, due to the limited availability of many protein standards, most protein assays utilize BSA or immunoglobulin as a calibration standard.[8] While the accompanying literature for the Coomassie® Plus assay reported an overall greater variation in protein-to-protein response at high concentrations, in the working region of 1 to 25 mg/L this variation appeared to be minimal. An experiment was conducted to evaluate the protein-to-protein response variation among four different proteins in PBS. Variation in protein-to-protein response was determined on standard solutions of BSA, lysozyme, ricin, and SEB prepared in PBS. Details are provided in Section 3.

2.6.3 Assay Repeatability.

Assay repeatability, or within-run precision, is a measure of the analysis precision obtained by a single operator, in one laboratory, using the same equipment, on the same occasion.[20] The acceptance criteria for this measure of precision depends on the analyte and the matrix in which it is analyzed. For example, a precision of $\leq$ 20% relative standard deviation (RSD) is acceptable in most environmental analyses.[21]

Assay repeatability was determined on two standard solutions of BSA and a PBS solution blank. The BSA standards were 0.8 and 12.5 mg/L, representing the estimated detection limit, and a mid-range calibration value. The BSA standards and PBS solutions were freshly prepared, then stored in 50 mL polypropylene sample bottles. The bottles were stored under ambient temperature (22-25 °C) and light conditions. An initial aliquot was analyzed immediately (time = 0), and 10 additional aliquots were then periodically withdrawn over the course of approximately 5 hr.

2.6.4 Assay Reproducibility.

Assay reproducibility, or between-run precision, is a measure of the analysis precision obtained by a single operator, but on different occasions.[22] The acceptance criteria for this measure of precision depends on the analyte and the matrix in which it is analyzed. For example, a precision of $\leq$ 20% RSD is acceptable in most environmental analyses.[21]

Assay reproducibility was determined using standard solutions of BSA, prepared from two independent primary stock solutions of BSA. A total of three concentrations; 0.781, 12.5, and 25.0 mg/L were prepared and analyzed to investigate assay reproducibility. These concentrations were chosen to cover the linear range, and are an estimate of the assay detection limit, the mid-point of the linear range, and the upper limit of the linear range. This evaluation was a sub-set of another experiment (Section 2.6.7), validating the calibration model. Standard solutions were prepared seven times from each primary stock, and were prepared and analyzed over a 5-day time period. The order of preparation, relative to primary stock, was randomized using a random number table.[20]

2.6.5 Assay Accuracy.

Accuracy of analytical measurements is defined in several different ways by various regulatory agencies. In general, accuracy is defined as the degree to which a measured value approaches its true value, and is most often expressed as percent recovery.[22]

Assay accuracy was determined by spiking BSA into buffer and applying the analysis assay. A total of seven independent preparations at BSA concentrations of 0.391, 0.781, 1.56, 3.12, 6.25, 12.5, and 25.0 mg/L were prepared and analyzed. Different primary stock solutions of BSA were used to generate the calibration curve and sample spiking solutions; each primary stock was obtained from a different source. Percent recoveries were then calculated relative to the established external calibration curve.

Assay accuracy was also determined by comparing results obtained from this assay with results obtained by an established assay. The concentration of a pure protein solution can be accurately determined by UV absorbance, if the extinction coefficient is known for the target analyte.[6] It follows from Beer's Law that the protein concentration can be calculated based upon the absorbance measurement at a specified wavelength, the path length of the sample, and the extinction coefficient particular to that protein at that specific wavelength, according to the following equation:

$$\text{Conc.} = A/(\varepsilon \times l)$$

where

Conc. = concentration of solution,
A = absorbance at specified wavelength,
ε = extinction coefficient, and
l = path length.

Assay accuracy was determined following this approach using a solution of the protein, SEB, with a known extinction coefficient of 1.4 for a 0.1% solution with path length of 1 cm at a wavelength of 277 nm.[23] As part of another study, SEB solutions were prepared and stored at 25 °C and monitored for protein stability.[10] At various time points, aliquots of solution were removed from the sample, quick frozen and stored for later analysis by the total protein assay and the UV method mentioned above. The samples were thawed, appropriately diluted to within the range of each analytical assay and analyzed. For total protein, the assay described in Section 3.1.9 was carried out. For the UV analysis, 180 µL of the diluted solution was transferred to a 0.1 cm quartz cuvette and the absorbance at 277 nm was recorded using a Jasco 810 spectropolarimeter. For blank subtraction, an aliquot of the diluent without protein was also measured by UV at 277 nm. The concentrations of the solutions were calculated from the results of each assay and compared to each other.

2.6.6 Matrix Interference.

Three potential sample matrices, DIW, STW, and CLW were evaluated for compatibility with this assay. In addition, the possible interference by sodium azide (NaN_3, CAS No. 26628-22-8) was also evaluated, as NaN_3 is used by the vendors as a preservative in stock solutions of ricin and BSA. This evaluation consisted of background absorbance measurements of the sample matrices, with and without Coomassie® Plus reagents, comparison of linear response of BSA in the potential sample matrices to the phosphate buffer, and influence of varying levels of NaN_3 on determined BSA concentrations over time.

The first evaluation focused on the background absorbance of each water matrix, with and without reagent added, as compared to the standard phosphate buffer. In this experiment, 300 µL of sample matrix was added to each of 48 wells of a plate, and 150 µL of sample matrix was added to each of the remaining 48 wells of the plate. Then, 150 µL of room

temperature Coomassie® Plus Reagent was added to each of the 48 wells containing 150 µL of sample matrix. Each plate was then analyzed in accordance with the procedures described in Section 3.1.9. In addition, pH was determined on each of the sample matrices, with and without the reagent added.

The second evaluation focused on the linearity of response of BSA prepared in each of the three potential sample matrices. Using the procedures described in Section 3.1.9, independent replicates of BSA calibration solutions were prepared in DIW, STW, and CLW. In addition, seven independent replicates of BSA calibration curves were also prepared and analyzed.

The third evaluation focused on the potential for NaN_3 to interfere with this assay. Sodium azide is a preservative commonly added to stock solutions of ricin and BSA. This evaluation was a sub-set of another experiment (Section 2.7), examining fate of stored BSA solution. A 15.0 mg/L solution of BSA (with 7.8 mg/L NaN_3) in STW was prepared, and 69 mL was placed into each of six 125 mL polypropylene bottles. An aliquot of STW and a stock solution of NaN_3, in varying proportions totaling 6 mL, were then added to each bottle. The final BSA concentration was 13.9 mg/L, and the NaN_3 concentrations were 3.5, 50, 100, 250, 500, and 800 mg/L. The bottles were stored at 40 °C, and periodically sampled. Samples were analyzed in accordance with the procedures described in Section 3.1.9.

2.6.7 External Calibration Model.

The external calibration model was established by preparation and analysis of BSA calibration standards in accordance with the procedures contained in Section 3.1.9. A total of 14 sets of calibration solutions were prepared and analyzed from two separate stock solutions of BSA from two separate vendors. The curves were generated over a five day period in a randomly assigned order, established by use of a random number table.[20] Seven curves were prepared from each stock. Each set of calibration standards consisted of a total of eight concentrations covering the range of 0 to 25 mg/L (0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25.0 mg/L) of BSA serially diluted in phosphate buffered saline solution. In all cases, the measured absorbance value of the associated assay blank (0 mg/L) was subtracted from the measured absorbance value of each individual corresponding calibration standard. Therefore, the 0 mg/L data were not included in the regression models.

2.6.8 Method Limits of Detection and Quantitation.

Method limits of detection and quantitation were determined according to an accepted approach in which analysis is carried out for samples containing the target analyte at a minimum of three different concentrations.[22] These analyses were performed at least seven times at each concentration level, as required. After the samples were analyzed, the standard deviation (SD) was calculated for each concentration level. The SD was then plotted against analyte concentration, and a linear regression model used to estimate the Y-intercept. The Y-intercept is referred to as sigma (S_0). The MLOD, at the 95% confidence interval, was then calculated as $3(S_0)$, and the MLOQ was calculated as $10(S_0)$.

2.6.9 Optimized Assay Parameters.

For the optimized assay, all solutions including blanks, samples, standards and the Coomassie® Plus Protein Assay Reagent were allowed to equilibrate to room temperature prior to transferring solutions to the to microplate.

One hundred fifty microliters of each blank, standard and/or sample solution to be analyzed was transferred to an empty well of the microplate. Depending on the volume of sample solution available, samples were either first transferred to reagent reservoirs for use with a multi-channel pipetter or pipetted singly into individual wells of the microplate. Prior to discharging the pipet contents into the well plate, the liquid level in each pipet tip was visually inspected to ensure that the sample volumes were consistent.

One hundred fifty microliters of Coomassie® Plus Protein Assay Reagent was added to the 150 µL of solution already contained in each well. The microplate was covered and immediately placed in the pre-heated 37 °C incubator of the microplate reader. The timer was immediately set for 10 min. The plate was shaken in the reader for approximately 30 sec and then allowed to incubate in the reader with the door closed. After the 10 min incubation period had elapsed, the door was opened, the lid was removed from the microplate, and the wells visually inspected for air bubbles. If no bubbles were observed, the endpoint absorbance measurement at 595 nm was immediately initiated. If air bubbles were observed, the pointed tip of a microliter syringe was used to remove them prior to the absorbance measurements.

2.7 Demonstration of Total Protein Assay.

Demonstration of the total protein assay was carried out during studies to determine the "fate" or stability of BSA in synthetic tap water when stored at two different temperatures for 28 days. A 15.0 mg/L solution of BSA (containing 7.8 mg/L NaN_3) was prepared in STW and an initial total protein measurement of the solution was made and evaluated against a freshly prepared six-point calibration curve. Following this initial analysis, 1.5 mL aliquots of the solution were transferred to each of 28 individual low protein binding, siliconized microcentrifuge tubes. Fourteen of these tubes were placed in an incubator at 25 °C, and the other 14 were incubated at 40 °C. Approximately 100 mL of the original 15 mg/L BSA solution were passed through a 0.22 µm sterilizing, low protein binding, PES filter membrane system. The filtered solution was also aliquoted into microcentrifuge tubes and incubated under the same conditions previously described above.

At various time points throughout the 28-day period, one microcentrifuge tube of unfiltered and filtered BSA solution from each of the temperature conditions was removed from the incubator and assayed for total protein following the assay described in Section 3.1.9. Three days were randomly selected, using a random number table,[20] to have duplicate tubes analyzed. In addition to these stability samples, a freshly prepared 15 mg/L BSA control sample was also analyzed to verify that the sample analysis was still in good agreement with the initial calibration curve.

An evaluation of the amount of total protein found in each solution at each time point when compared with the initial total protein measurement provides an indication as to the stability of the BSA in solution with regard to protein denaturation, precipitation and adherence to the container walls.

3. RESULTS AND DISCUSSION

3.1 Assay Optimization and Validation.

3.1.1 Reaction Time.

The results are illustrated in Figure 1 and compare the absorbance measurements obtained for BSA (12.5 mg/L) in DIW, STW, and CLW relative to absorbance measurements obtained in the baseline PBS. The decrease in absorbance appears to follow zero order kinetics and half-lifes ($t_{1/2}$) were calculated using a zero order model. The regression parameters and calculated half-lifes are summarized in Table 4. In calculating the $t_{1/2}$ values, the average (n=3) initial absorbance value was used for each matrix. The average initial absorbance values were 0.2560, 0.2471, 0.2697, and 0.2286 for the PBS, DIW, STW, and CLW matrices, respectively. The small number of replicates (n=3) prevents a rigorous statistical comparison of initial absorbance values, but the initial absorbance value for the BSA prepared in CLW is lower than when prepared in the other three matrices. This is, most likely, due to the reaction of free chlorine with the reagents used in the assay. The overall $t_{1/2}$ was calculated to be 118 min (n = 12, SD = 17.9, RSD = 15.2%), with no apparent differences between sample matrices. Again, the small number of replicates (n=3) for each matrix prevents a rigorous statistical comparison. The vendor noted the absorbance measurements should be taken after 10 min of incubation to obtain consistent results.[8] These experiments demonstrate that the intended sample matrices do not differ significantly from results obtained in PBS, and 10 min of incubation time was used for all the sample matrices considered in this effort. ***Consistent and accurate timing is critical to obtaining good precision with this assay.***

3.1.2 Individual Protein Response.

Variation in protein to protein response was determined on standard solutions of BSA, lysozyme, ricin, and SEB prepared in PBS solution using a serial dilution approach. A total of 11 concentrations, ranging from 0.5 to 150 mg/L, were evaluated for each individual protein. Each concentration series was independently prepared, just prior to analysis. Absorbance for each solution was measured in eight wells, with the average absorbance for each solution compared to the overall BSA absorbance. In all cases, sample absorbance values were corrected for the background absorbance of phosphate buffer. There were no anomalies during the preparation or analysis of these sample solutions. The data are illustrated in Figures 2 through 4 and demonstrate that lysozyme and SEB give comparable absorbance responses as those obtained with BSA. While ricin exhibits linear behavior, the sensitivity of response is significantly different from that of BSA. The regression parameters and percent difference of sensitivities, relative to BSA, are summarized in Table 5. The absorbance values become non-linear after approximately 25 mg/L and demonstrate saturation of response. This saturation,

most likely, is due to consumption of available reagent. The results of this experiment demonstrate BSA is a suitable surrogate standard for lysozyme and SEB, but is not a suitable surrogate for ricin.

3.1.3 Assay Repeatability.

Results of the assay repeatability study are illustrated in Figure 5. A total of 16 wells were prepared and analyzed for every time point collected from the standard solutions, for a total of 176 absorbance values. Only eight wells were prepared and analyzed for the first two time points collected from the blank solution, and one well from the 60 min time point was not used due to formation of a bubble in the well. A total of 159 absorbance values were collected during analysis of the blank solution.

The results obtained demonstrate the assay is repeatable over the time frame evaluated, which was a typical working day. The percent RSD values were 2.32% for the blank solution, 1.88% for the 0.8 mg/L BSA standard, and 2.36% for the 12.5 mg/L BSA standard. These RSD values are well below the 20% RSD typically found to be acceptable in environmental analyses.[21]

3.1.4 Assay Reproducibility.

Results of the assay reproducibility study are summarized in Table 6. A total of 112 wells were analyzed for the 12.5 and 25.0 mg/L solutions. Only 111 wells were measured for the 0.781 mg/L solution, as a bubble developed in one of the wells. The percent RSD values were 100% for the 0.781 mg/L BSA standard, 5.15% for the 12.5 mg/L standard, and 4.80% for the 25.0 mg/L standard. The RSD values for the 12.5 and 25.0 mg/L standards are well below the 20% RSD typically found to be acceptable in environmental analyses.[21]

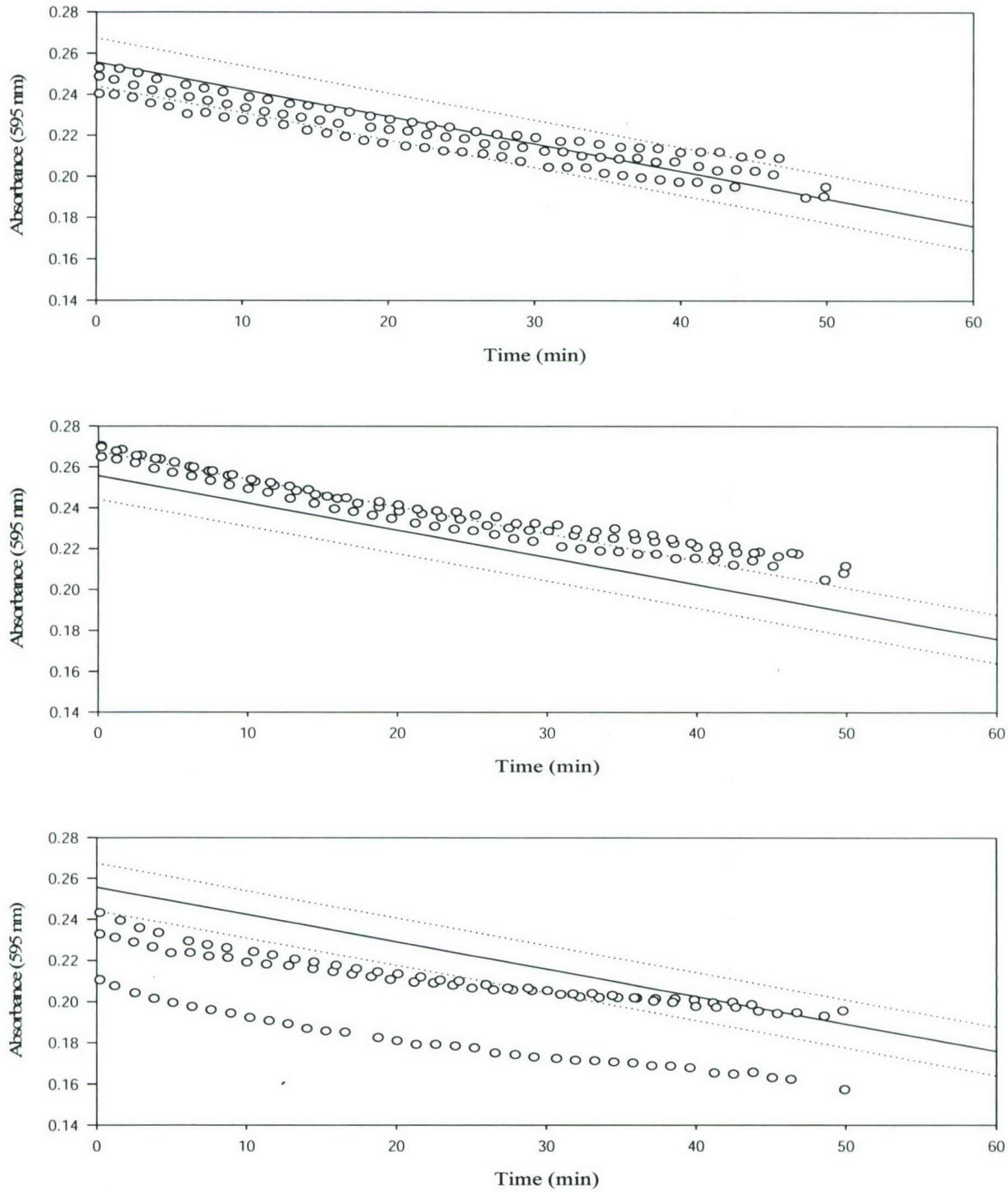

Figure 1. Reaction Product Stability as a Function of Time at 37 °C. Data is shown for the BSA standard prepared in DIW (upper panel), in STW (middle panel), and in CLW (bottom panel). The solid black line is the linear regression line of the BSA standards prepared in PBS solution, and the dotted lines are the 95% prediction interval about the regression line. Each panel shows the data for three independent replicates (three sets of open circles per panel).

Table 4. Summary of Regression Parameters and Calculated Half-Lifes of the Reaction Product Absorbance Over Time

Aqueous Matrix	Replicate Number	Regression Parameters[a]			Calculated $t_{1/2}$ (min)[b]
		m	b	r^2	
PBS	1	-0.001452	0.2535	0.9948	88.1
	2	-0.001376	0.2640	0.9850	93.0
	3	-0.001184	0.2505	0.9853	108
DIW	1	-0.001029	0.2380	0.9909	120
	2	-0.0009489	0.2490	0.9692	130
	3	-0.0009917	0.2442	0.9835	124
STW	1	-0.001192	0.2608	0.9750	113
	2	-0.001151	0.2650	0.9680	117
	3	-0.001157	0.2700	0.9855	117
CLW	1	-0.0007192	0.2272	0.9550	159
	2	-0.0009514	0.2350	0.9622	120
	3	-0.0009431	0.2029	0.9631	121

a. Used a linear model; $Y = mX + b$.

b. Calculated using a zero order model; $t_{1/2}$ = (initial absorbance/2(k)), where k = slope.

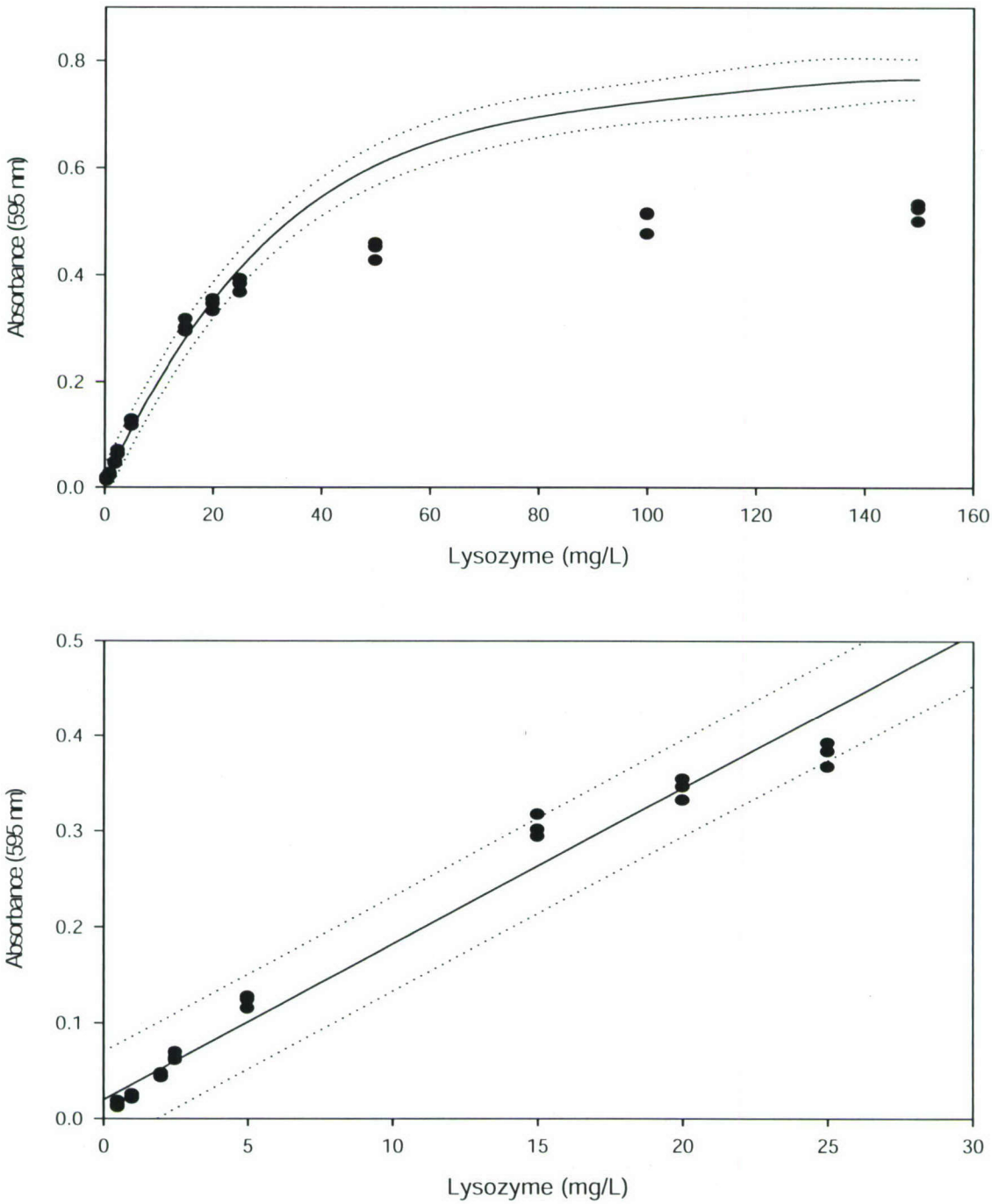

Figure 2. Response Curves for Lysozyme as Compared to BSA. The upper panel is the full range evaluated, and the bottom panel is the estimated linear response range. The solid black line is the regression curve for BSA, and the dotted lines delineate the 95% prediction interval about the BSA regression line. Each panel shows the data for three independent replicates (three sets of closed circles per panel).

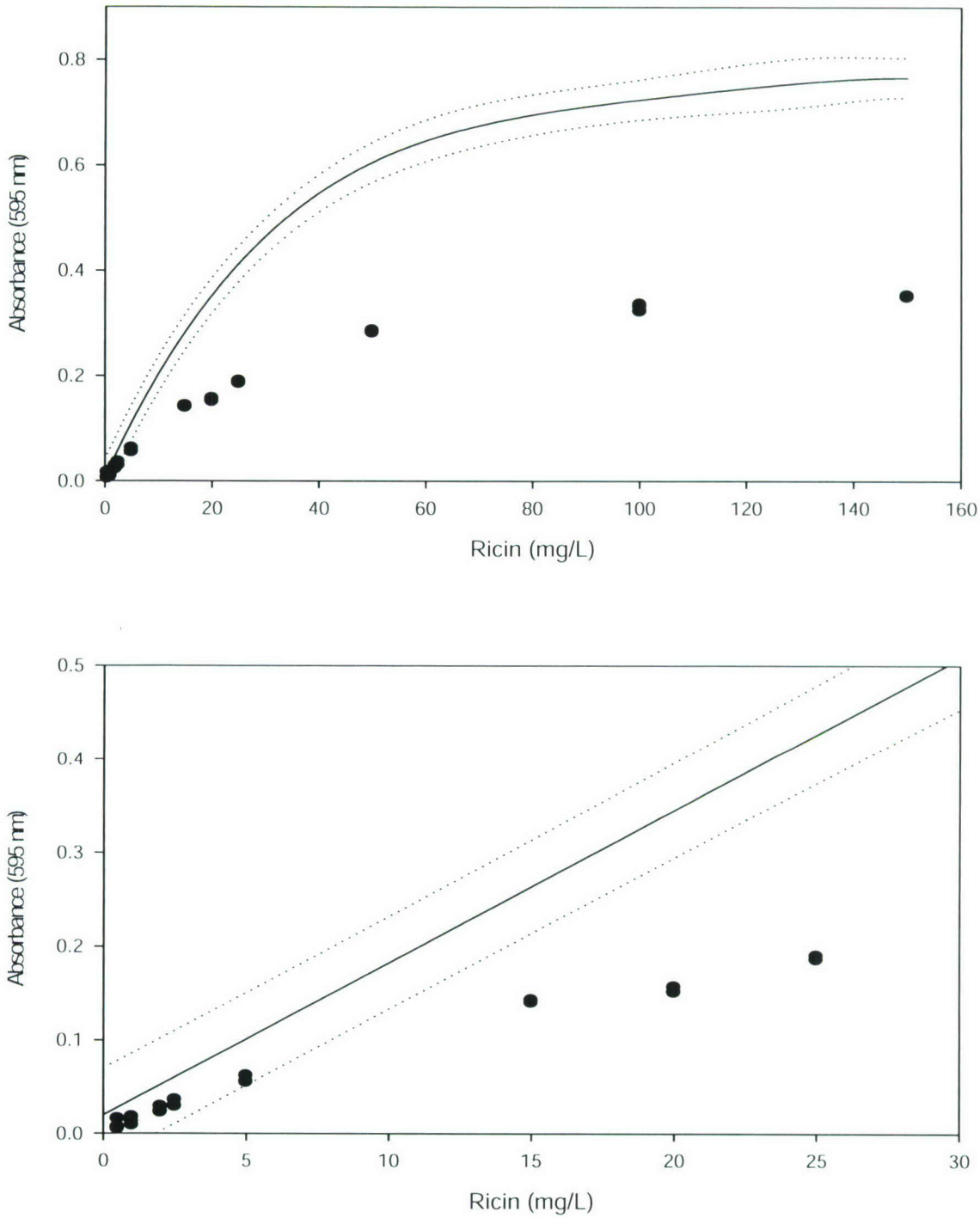

Figure 3. Response Curves for Ricin as Compared to BSA. The upper panel is the full range evaluated, and the bottom panel is the estimated linear response range. The solid black line is the regression curve for BSA, and the dotted lines delineate the 95% prediction interval about the BSA regression line. Each panel shows the data for three independent replicates (three sets of closed circles per panel).

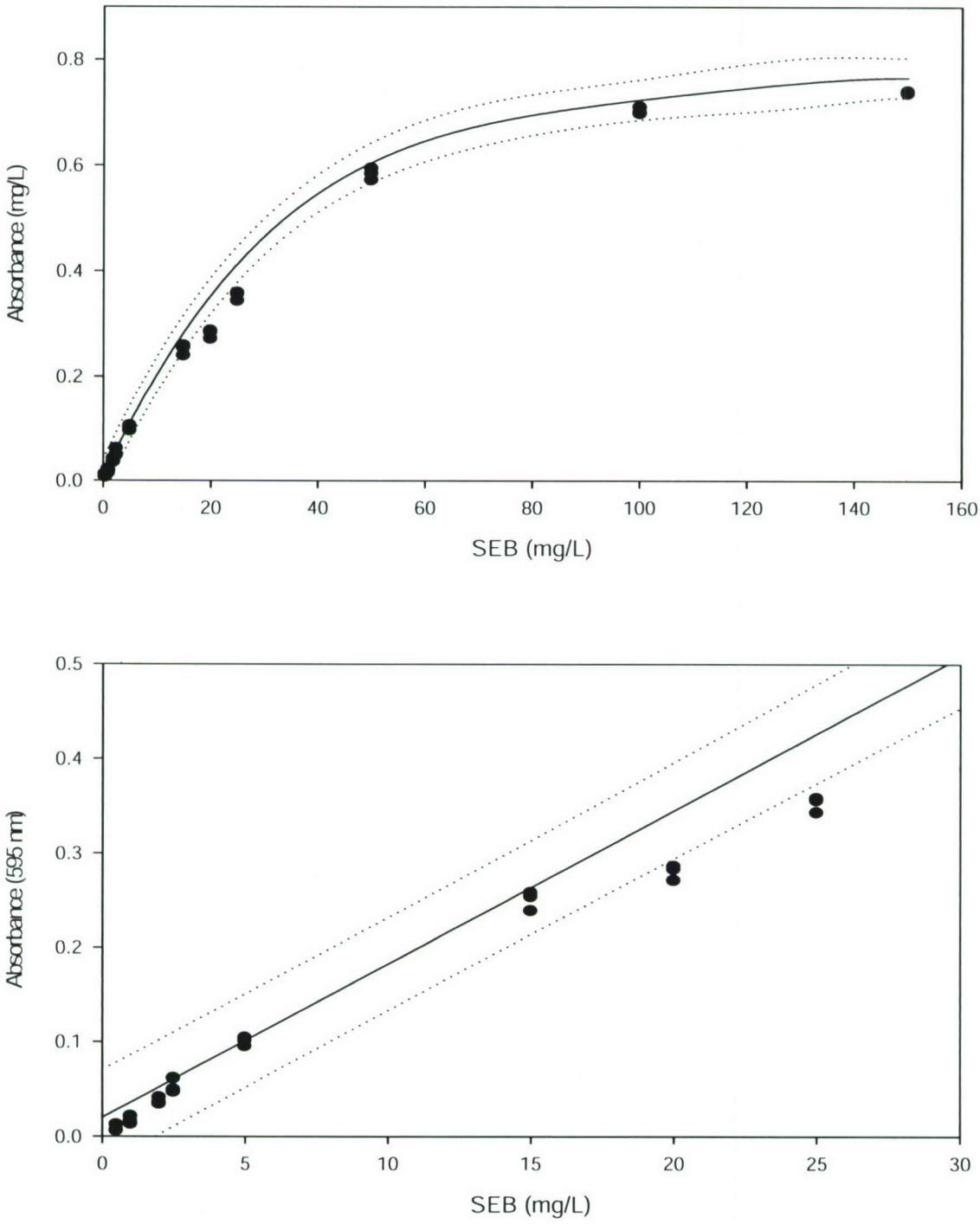

Figure 4. Response Curves for SEB as Compared to BSA. The upper panel is the full range evaluated, and the bottom panel is the estimated linear response range. The solid black line is the regression curve for BSA, and the dotted lines delineate the 95% prediction interval about the BSA regression line. Each panel shows the data for three independent replicates (three sets of closed circles per panel).

Table 5. Summary of Regression Parameters and Percent Difference of Sensitivities of Protein Toxins Relative to BSA

Protein Standard	Regression Parameters[a]			Percent Difference[b]
	m	B	r^2	
BSA	0.01627	0.01974	0.9766	0 %
Lysozyme	0.01575	0.02237	0.9719	3.25 %
SEB	0.01495	0.01328	0.9858	8.46 %
Ricin	0.007357	0.01225	0.9816	75.4 %

a. Used a linear model; $Y = mX + b$.
b. Percent difference relative to sensitivity of BSA.

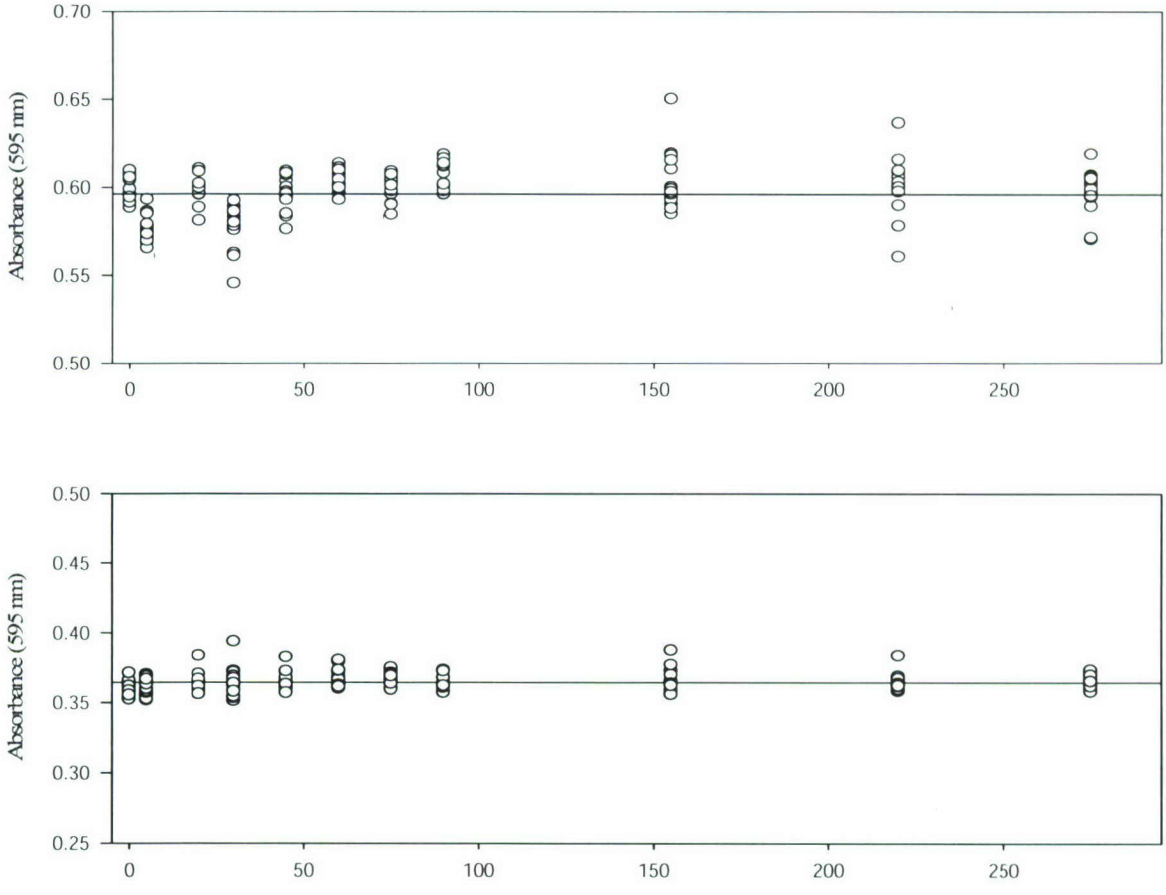

Figure 5. Assay Repeatability as a Function of Absorbance at 595 nm. The upper panel is the 12.5 mg/L BSA standard, the middle panel is the 0.8 mg/L BSA standard, and the bottom panel is the phosphate buffered saline blank solution. No background subtraction was performed, and the horizontal black lines are the average absorbance values. Each panel shows the data for sixteen measurements per time point (sets of open circles). (Figure is continued on next page.)

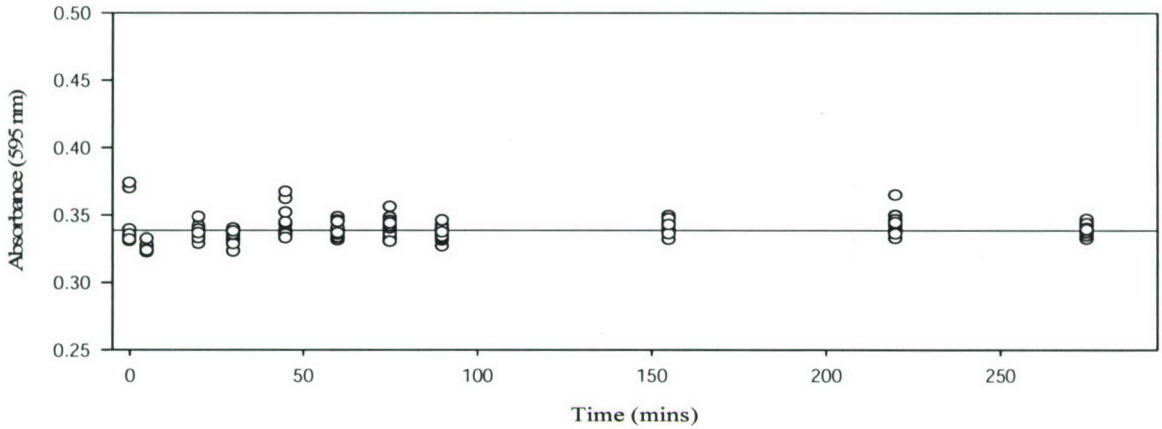

Figure 5. Assay Repeatability as a Function of Absorbance at 595 nm. The upper panel is the 12.5 mg/L BSA standard, the middle panel is the 0.8 mg/L BSA standard, and the bottom panel is the phosphate buffered saline blank solution. No background subtraction was performed, and the horizontal black lines are the average absorbance values. Each panel shows the data for sixteen measurements per time point (sets of open circles). (Figure is continued from previous page.)

Table 6. Statistical Summary from the Reproducibility Experiment with Absorbance Corrected for the Buffer Blank

BSA	Absorbance @ 595 nm			
(mg/L)	N	Mean	SD	% RSD
0.781	111	0.0103	0.0103	100
12.5	112	0.2174	0.0112	5.15
25.0	112	0.3960	0.0190	4.80

3.1.5 Assay Accuracy.

The recovery data from the BSA spiking experiment is summarized in Table 7. There were no anomalies during the preparation and analysis of these samples. The overall recovery for concentrations above the MLOQ was 99.6%, and there was no apparent correlation with BSA concentration. The recoveries for concentrations below the MLOQ (0.391 and 0.781 mg/L) were 41.8 and 67.5%, respectively.

Assay accuracy was also determined by comparing results obtained from this total protein assay, with results obtained by an established UV-based assay.[6] This comparison was performed on SEB samples prepared in two different water matrices, PB and CLW.

The comparison data are illustrated in Figure 6, and demonstrate good agreement between the two assays. Overall, the percent difference was 10.7 %, and there was no apparent bias in the total protein assay relative to the UV assay.

Table 7. Summary of Recovery Data

BSA Spike (mg/L)	Spike Recovery (%)			
	Range	Average	SD	% RSD
0.391	0 – 197	41.8	102	243
0.781	29.3 – 144	67.5	40.5	60.0
1.56	62.3 – 128	88.9	20.7	23.3
3.12	90.0 – 124	106	11.2	10.6
6.25	91.9 – 122	107	9.28	8.67
12.5	95.2 – 108	102	4.08	3.98
25.0	88.6 – 99.3	94.3	3.29	3.49

Spikes were performed in PBS solution, with seven independent serial dilutions prepared.

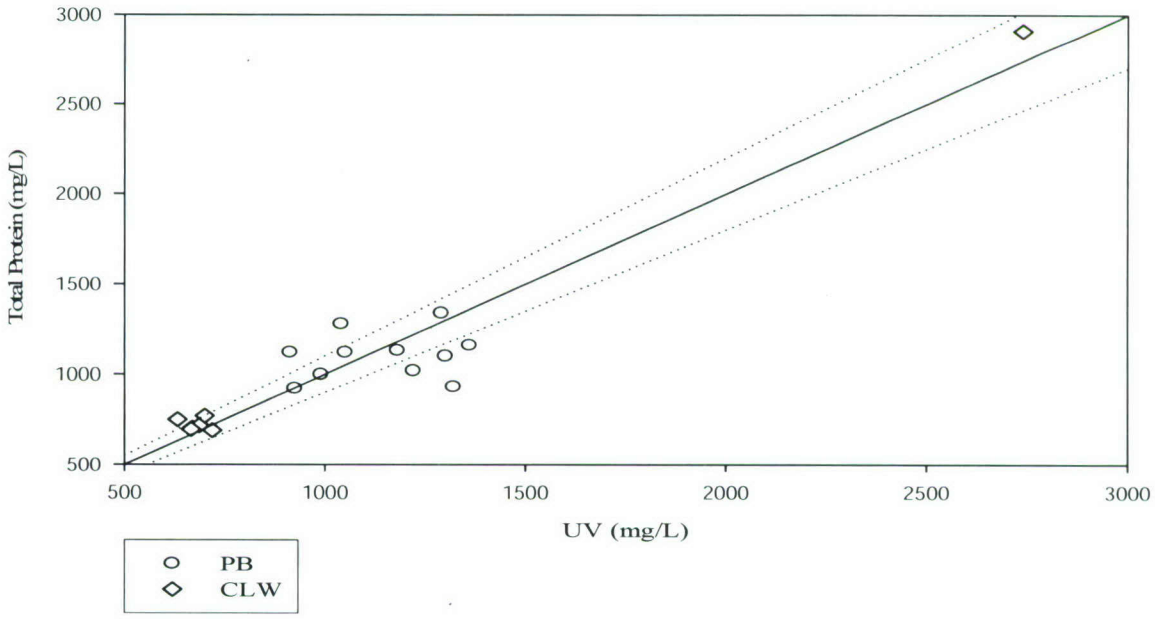

Figure 6. Comparison of Results from the Total Protein Assay and the Established UV Assay for Phosphate Buffer (PB) and Chlorinated Water (CLW). The solid line is the 1:1 line, and the dotted lines are ±10% from the 1:1 line.

3.1.6 Matrix Interference.

As noted under Section 2.6.6, three potential sample matrices (DIW, STW, and CLW) were evaluated for compatibility with this assay and the possible interference by NaN_3 was also evaluated.

The data for the background absorbance of each water matrix are summarized in Tables 8 and 9, and there was one anomaly during the analysis of these samples. In the mixture of DIW and Coomassie® Plus Reagent, only 45 absorbance values were collected out of 48 wells. Data from three of the wells was not used because there were bubbles in the sample wells, which affected the absorbance measurement. The mean absorbance values for each of the water matrices was compared to the mean absorbance of the phosphate buffer control, using a standard significance test.[22] In all cases of just the sample matrix (no reagent added), the calculated t-values were less than the critical t-value (DF = 50, P = 0.01) of 2.68, suggesting no significant difference in absorbance response. In the case of sample matrix mixed with Coomassie® Plus Reagent, the calculated t-values for DIW and STW were less than the critical t-value (DF=50, P= 0.01) of 2.68, suggesting no significant difference in absorbance response. The calculated t-value for the CLW mixed with Coomassie® Plus Reagent was greater than the critical t-value, indicating there was a significant difference between absorbance of the phosphate buffer control and the CLW sample. In fact, the average absorbance of the CLW sample was 19% less than the absorbance of the buffer control. The reason for this difference does not appear to be due to pH differences, as all the sample matrices mixed with reagent had approximately the same pH. The pH of the final mixtures was 0.94 for buffer, 0.97 for DIW, 0.98 for STW, and 0.98 for CLW. One difference noted in this experiment was the color of the sample solutions mixed with the reagent. The CLW mixed with the reagent was green, while all the other samples were blue. Apparently, the residual chlorine is reacting with the Coomassie® Plus Reagent.

Table 8. Absorbance Values (595 nm) Obtained on the Aqueous Test Matrices

Statistical Parameter	Aqueous Matrix			
	Buffer	DIW	STW	CLW
Mean	0.038	0.036	0.037	0.037
SD	0.0083	0.0018	0.0016	0.0020
% RSD	21.8	5.00	4.32	5.40
95% CI	± 0.0024	± 0.0005	± 0.0005	± 0.0006
N	48	48	48	48

Table 9. Absorbance Values (595 nm) Obtained on the Aqueous Test Matrices Mixed with Coomassie® Plus Reagent

Statistical Parameter	Aqueous Matrix with Reagent Added			
	Buffer	DIW	STW	CLW
Mean	0.320	0.319	0.324	0.261
SD	0.0244	0.0050	0.0090	0.0036
% RSD	7.63	1.57	2.78	1.38
95% CI	± 0.0035	± 0.0015	± 0.0026	± 0.0010
N	48	45	48	48

For the linearity of response of BSA prepared in each of the water matrices, there was one anomaly during the preparation and analysis of these samples; data from one well (DIW, 1.6 mg/L) was not used due to formation of a bubble. Two replicate curves were prepared in the DIW and STW matrices, and 3 replicate curves were prepared in the CLW matrix. The data are illustrated in Figure 7 and demonstrate that there are no significant differences between calibration curves prepared in DIW and STW as compared to calibration curves prepared in PBS solution. The curves prepared in CLW become non-linear after the 12.5 mg/L level. The reason for this is not known, but it is suspected that the residual chlorine in the CLW matrix is reacting with the Coomassie® Plus Reagent, leaving less reagent available to react with the protein.

The results for the evaluation of potential interference by NaN_3 are illustrated in Figure 8 and suggest there is no significant impact on the total protein results if NaN_3 is present in the sample matrix at the concentrations evaluated.

3.1.7 External Calibration Model.

The regression equations for the calibration curves obtained are summarized in Table 10, and example calibration curves are illustrated in Figures 9 and 10. In all cases, the measured absorbance value of the associated assay blank (0 mg/L) was subtracted from the measured absorbance value of each individual corresponding calibration standard. Therefore, the 0 mg/L data were not included in the regression models. The assay is linear in the range of 1-25 mg/L, and is in agreement with information provided by the vendor of the assay.[8] There were no significant differences observed in the sensitivity obtained for each source of BSA.

3.1.8 Method Limits of Detection and Quantitation.

The data for the determination of MLOD and MLOQ are illustrated in Figure 11, and S_0 was calculated to be 0.1167 mg/L. The MLOD was calculated to be 0.350 mg/L, and the MLOQ was calculated to be 1.17 mg/L.

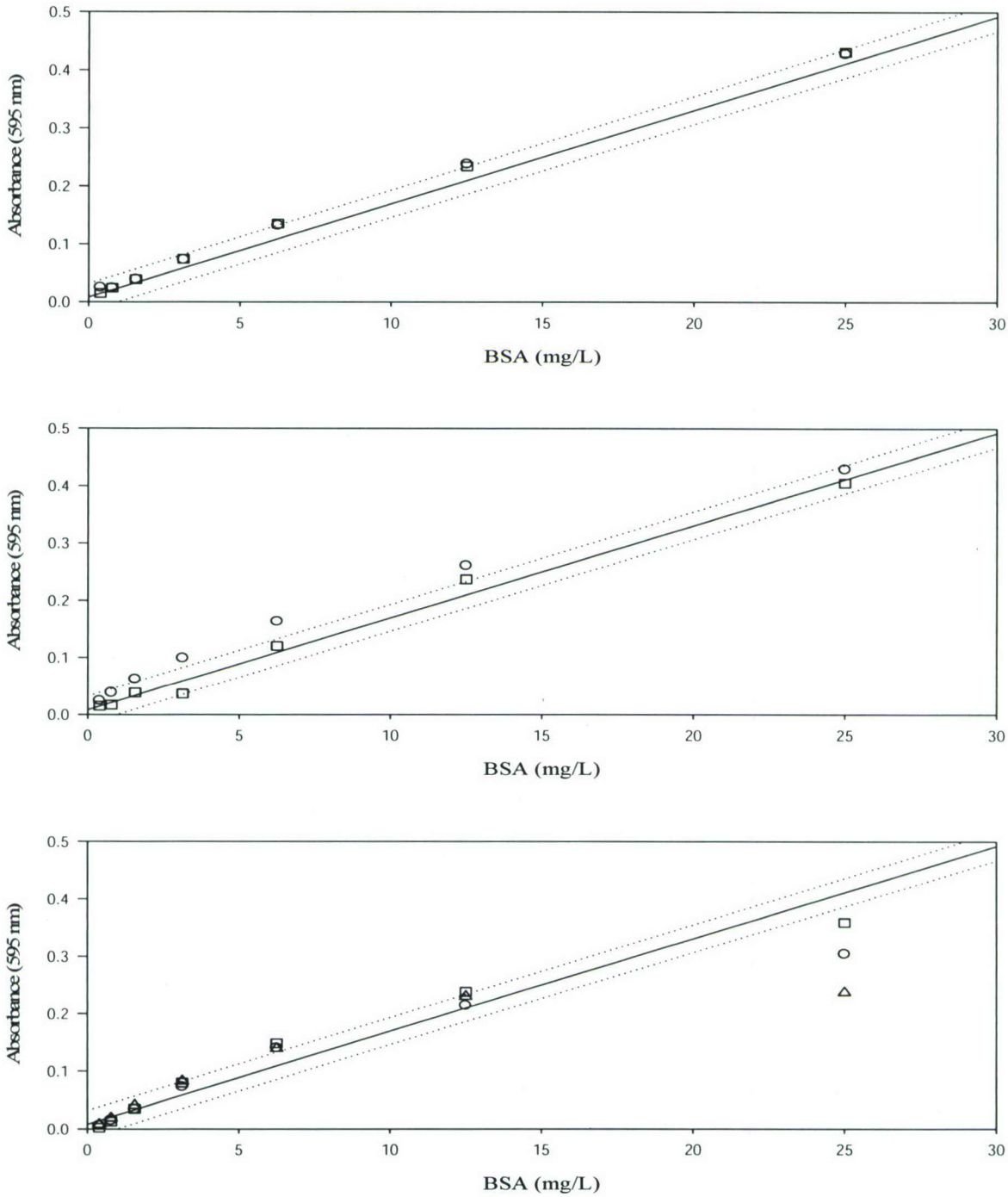

Figure 7. External Calibration Curves for BSA Prepared in Three Different Aqueous Matrices. The top panel is DIW, the middle panel is STW, and the bottom panel is CLW. The solid black line is the linear regression line for 7 separate preparations of BSA in buffer, and the dotted lines represent the 95% prediction interval about the regression line. Open circles are replicate one, open squares are replicate two, and open triangles are replicate three (CLW only).

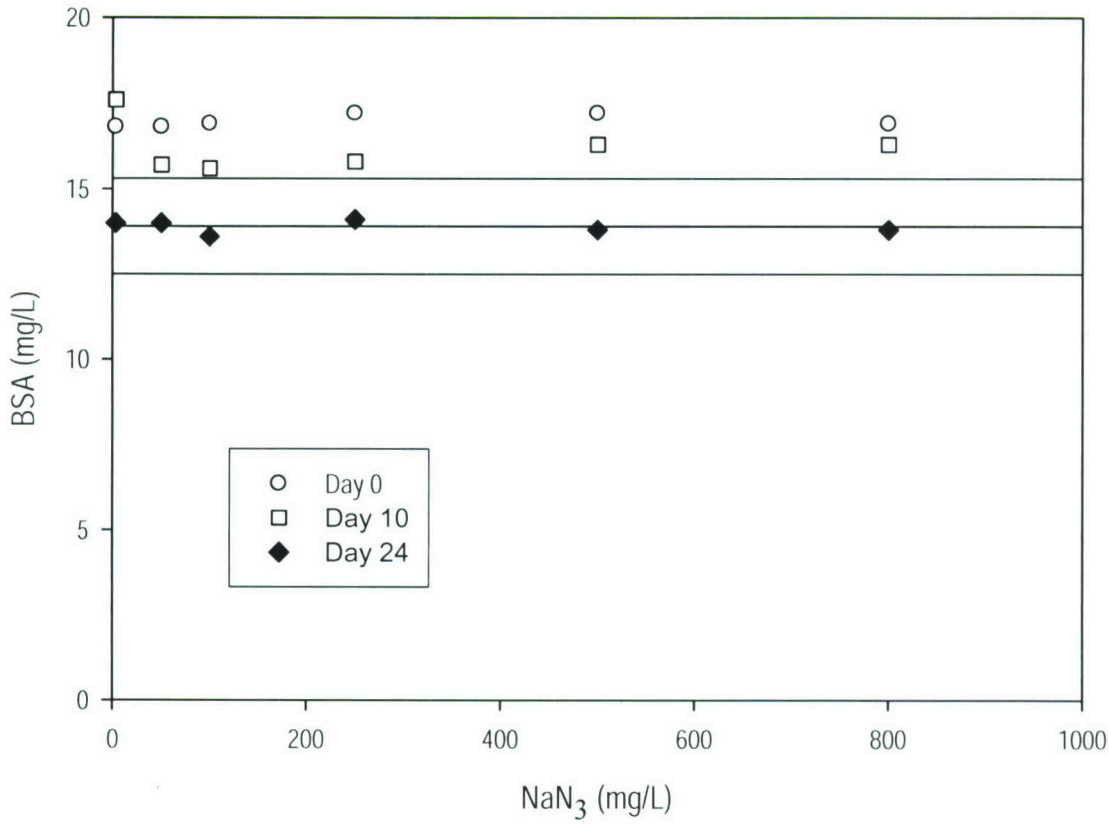

Figure 8. Measured BSA as a Function of NaN_3 Concentration. Bovine serum albumin was measured after storage of solution for three different time periods at 40 °C: 0, 10, and 24 days. The horizontal black lines represent the initial BSA concentration, and ± 10% of the initial BSA concentration.

Table 10. Summary of Linear Regression Parameters for Each of the 14 BSA Calibration Curves

BSA Source	Linear Regression Parameters			
	m	b	R^2	Average[a]
One[b]	0.0162	0.0005	0.9968	M = 0.0154 B = 0.0104 R^2 = 0.9935
	0.0156	0.0002	0.9982	
	0.0166	0.0073	0.9934	
	0.0172	0.0001	0.9988	
	0.0168	0.0071	0.9944	
	0.0168	0.0034	0.9981	
	0.0155	0.0066	0.9936	
Two[c]	0.0160	0.0066	0.9926	M = 0.0158 B = 0.00922 R^2 = 0.9925
	0.0164	0.0080	0.9947	
	0.0148	0.0030	0.9975	
	0.0158	0.0041	0.9963	
	0.0158	0.0033	0.9948	
	0.0159	0.0038	0.9967	
	0.0155	0.0037	0.9962	

a. Based on all data points.
b. BSA obtained from Pierce Chemical Company
c. BSA obtained from Sigma-Aldrich.

The BSA standards were analyzed in the range of 0.391 to 25.0 mg/L. The linear model is represented by y=mx+b.

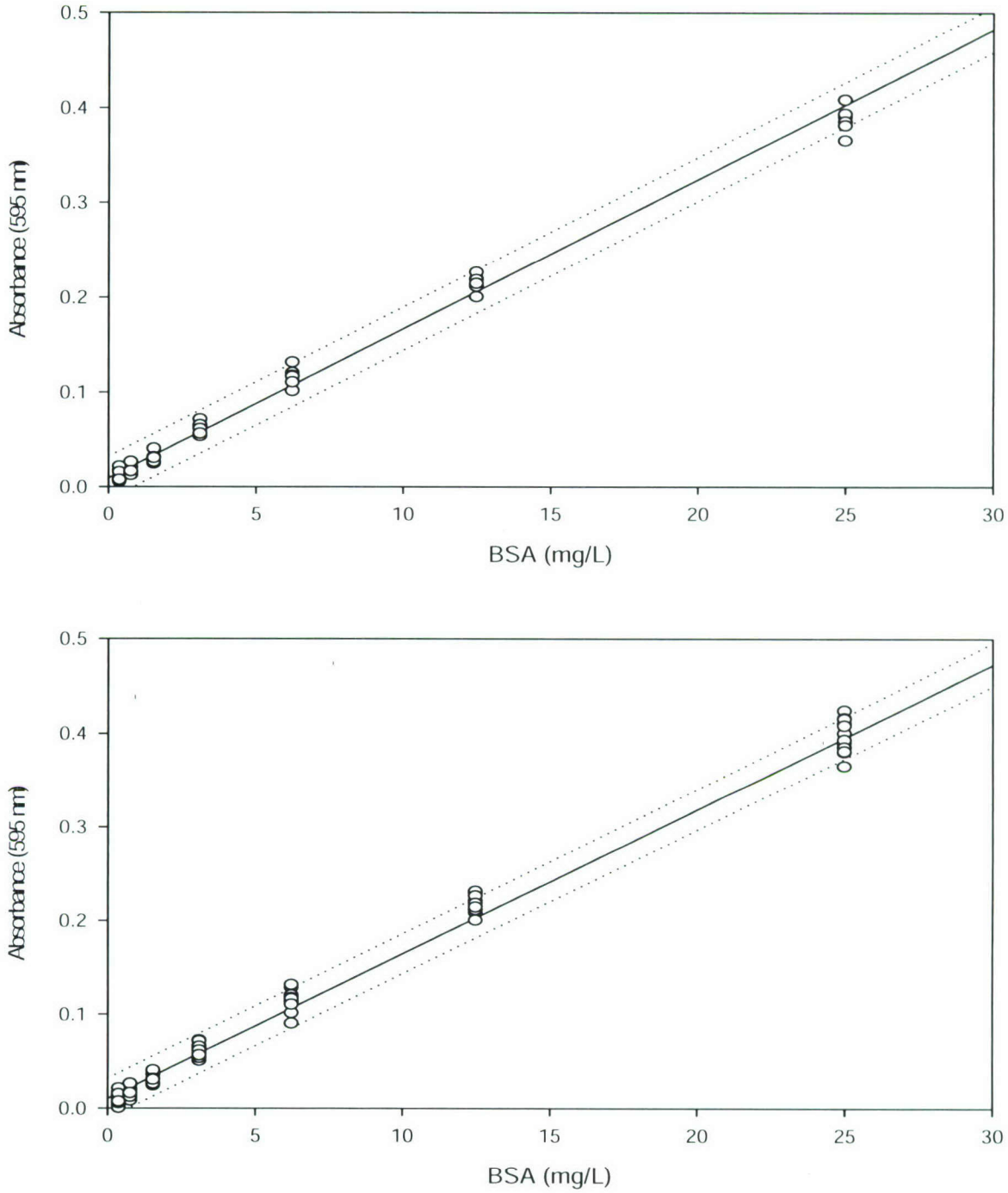

Figure 9. Comparison of BSA from Two Different Sources. The upper panel is the data from source two compared to the overall regression generated from source one BSA. The lower panel is the data from source one compared to the overall regression generated from source two BSA. The solid line is the linear regression line, and the dotted lines are the 95% prediction interval about the regression line. Fourteen separate sets of calibration solutions were prepared. Each open circle for each concentration represents the average of eight wells for one solution.

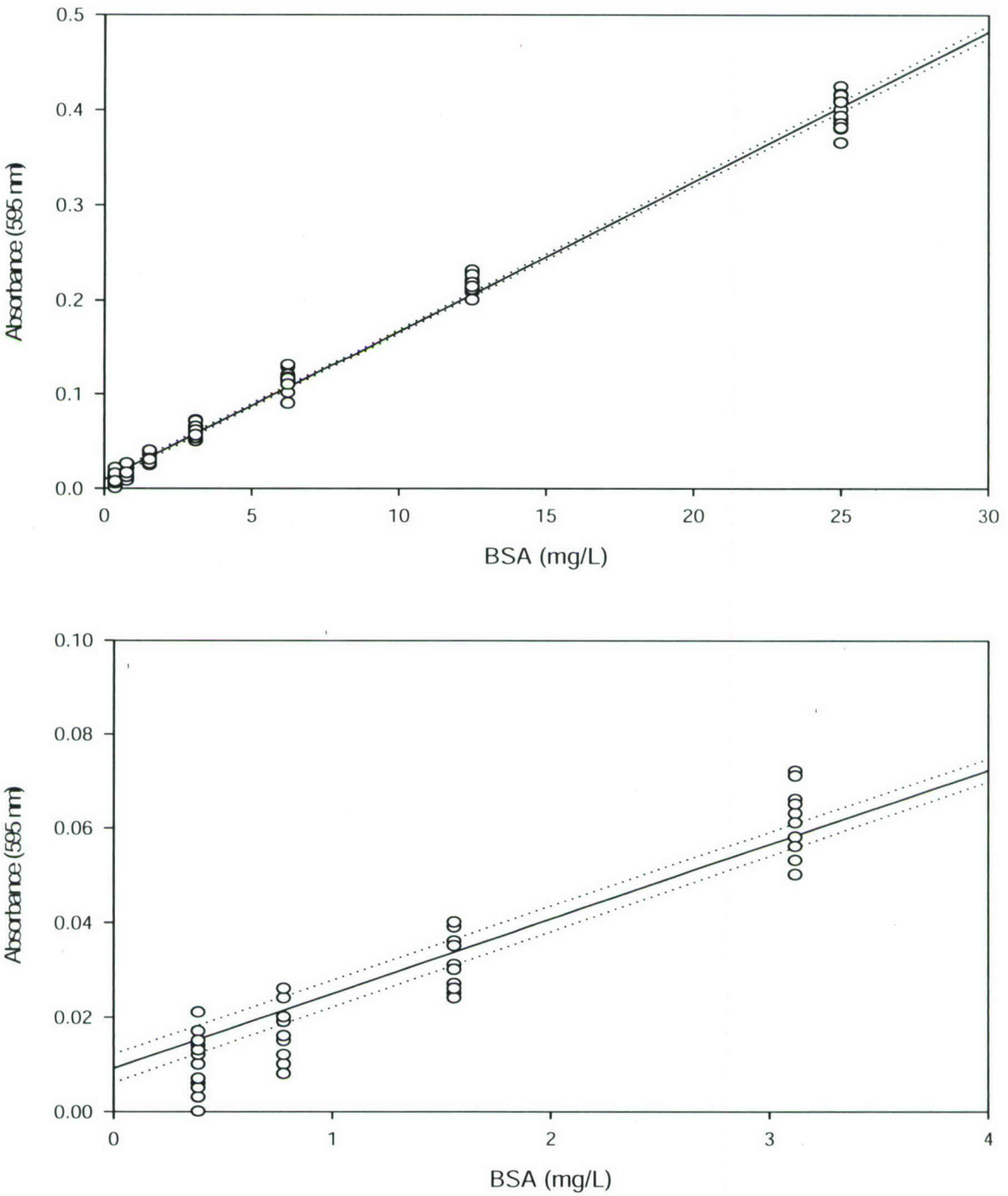

Figure 10. Overall External Calibration Model. The top panel is the full linear range, and the bottom panel is zoomed into the lower end of the curve. The solid line is the linear regression line, and the dotted lines are the 95% confidence interval about the regression line. Fourteen points per concentration, and each point is the average of eight absorbance measurements.

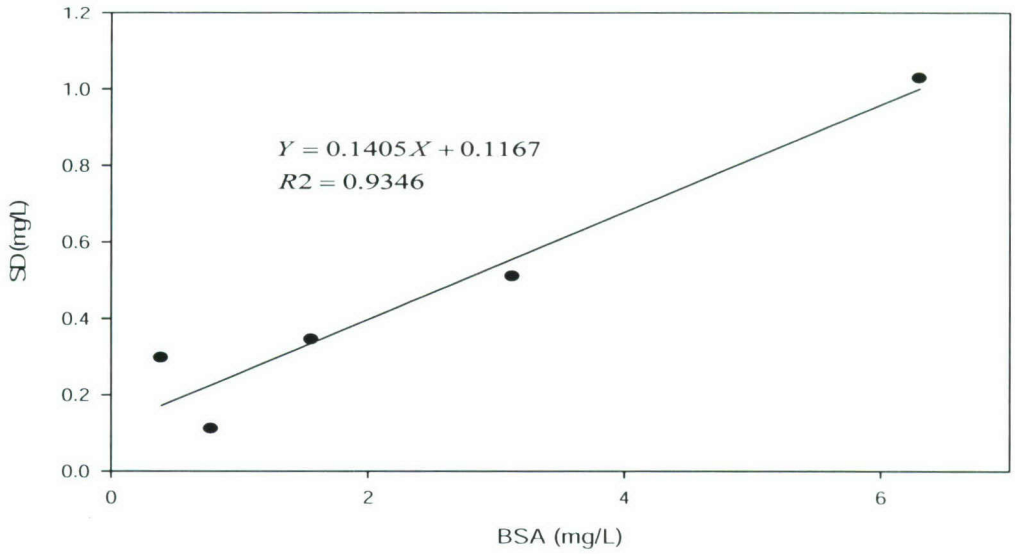

Figure 11. Determination of S_0 for the Calculation of MLOD and MLOQ

3.2 Demonstration of Total Protein Assay.

The results for the demonstration of the total protein assay for fate studies are illustrated in Figures 12 and 13 and demonstrate that BSA is stable under the storage conditions evaluated. One data point, day seven of the 25 °C treatment, was not used in the regression. The total protein assay is suitable for evaluating protein concentration in fate studies.

4. CONCLUSIONS

A quantitative assay for the determination of total protein in aqueous matrices has been successfully optimized and validated. The analysis time is less than 30 min, and the assay was adapted for implementation in a plate reader format to minimize sample and reagent volumes. The assay was linear over the range of 1 to 25 mg/L, though the ricin sensitivity was significantly different from the other proteins evaluated during this study. The method limit of detection was found to be 0.350 mg/L BSA, and the method limit of quantitation was found to be 1.17 mg/L BSA. The assay was found to be repeatable and reproducible, with RSD values typically below 5%. The assay was found to be accurate to within 10.7 %, with no apparent bias when compared to an established absorbance assay. Sodium azide did not, at the concentrations evaluated, interfere with this assay. The only sample matrix to exhibit potential interference with this assay was CLW. The low limit of detection, linear range, repeatability, reproducibility, relative responsiveness to individual proteins, and robustness in differing water matrices make the Coomassie® Plus Protein Assay suitable for biotoxin fate studies.

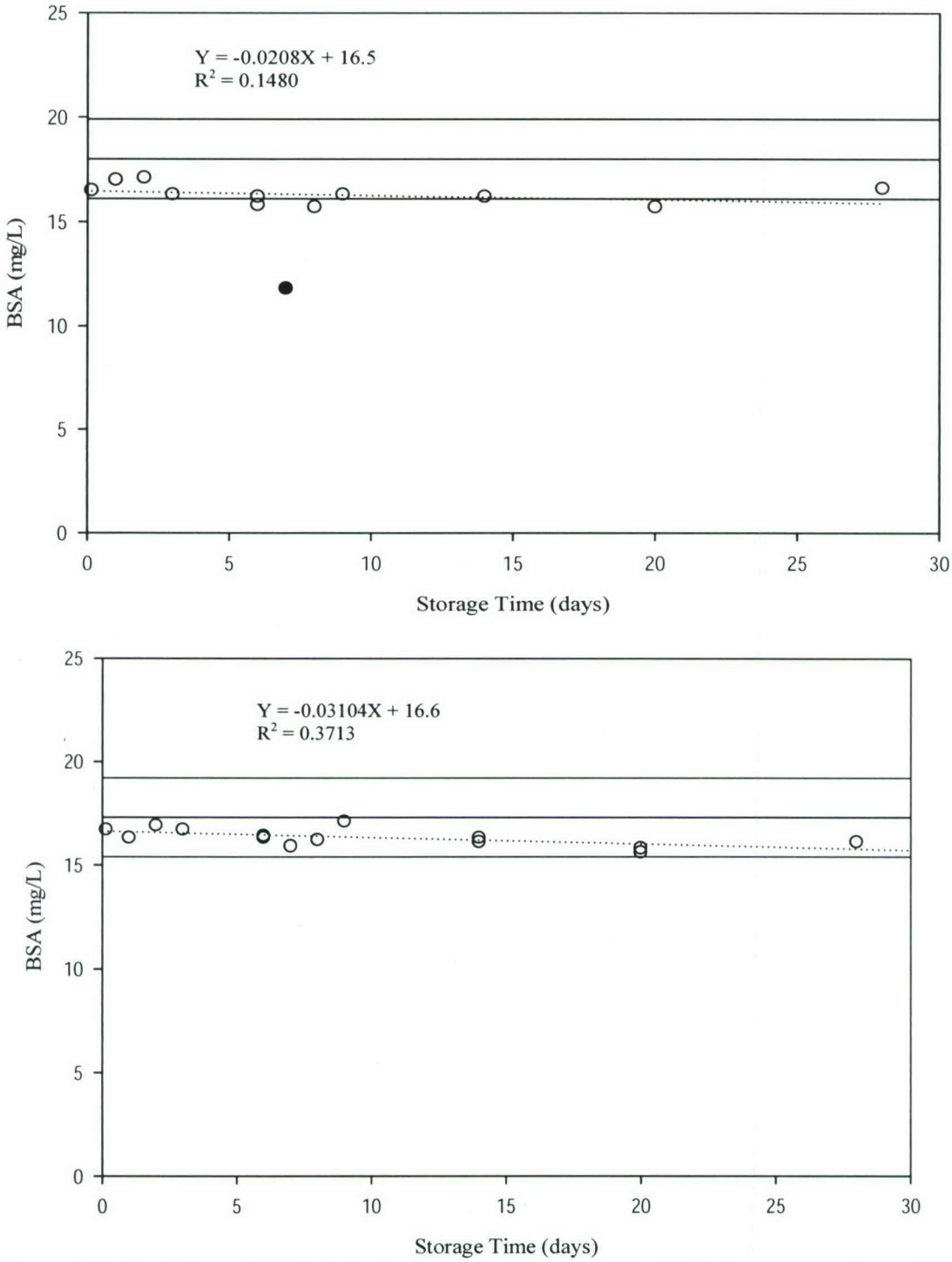

Figure 12. Stability of BSA Solution Stored at 25 °C. The initial concentration of BSA was 15 mg/L. The upper panel is the unfiltered sample, and the bottom panel is the filtered sample. The dotted line is the linear regression line and the solid lines are the average, minimum and maximum BSA concentration at time zero. The solid data point was not used in the regression calculations. Each open circle represents the average of eight measurements on one sample, with two samples being analyzed on days 6, 14, and 20 and single samples analyzed on the other days.

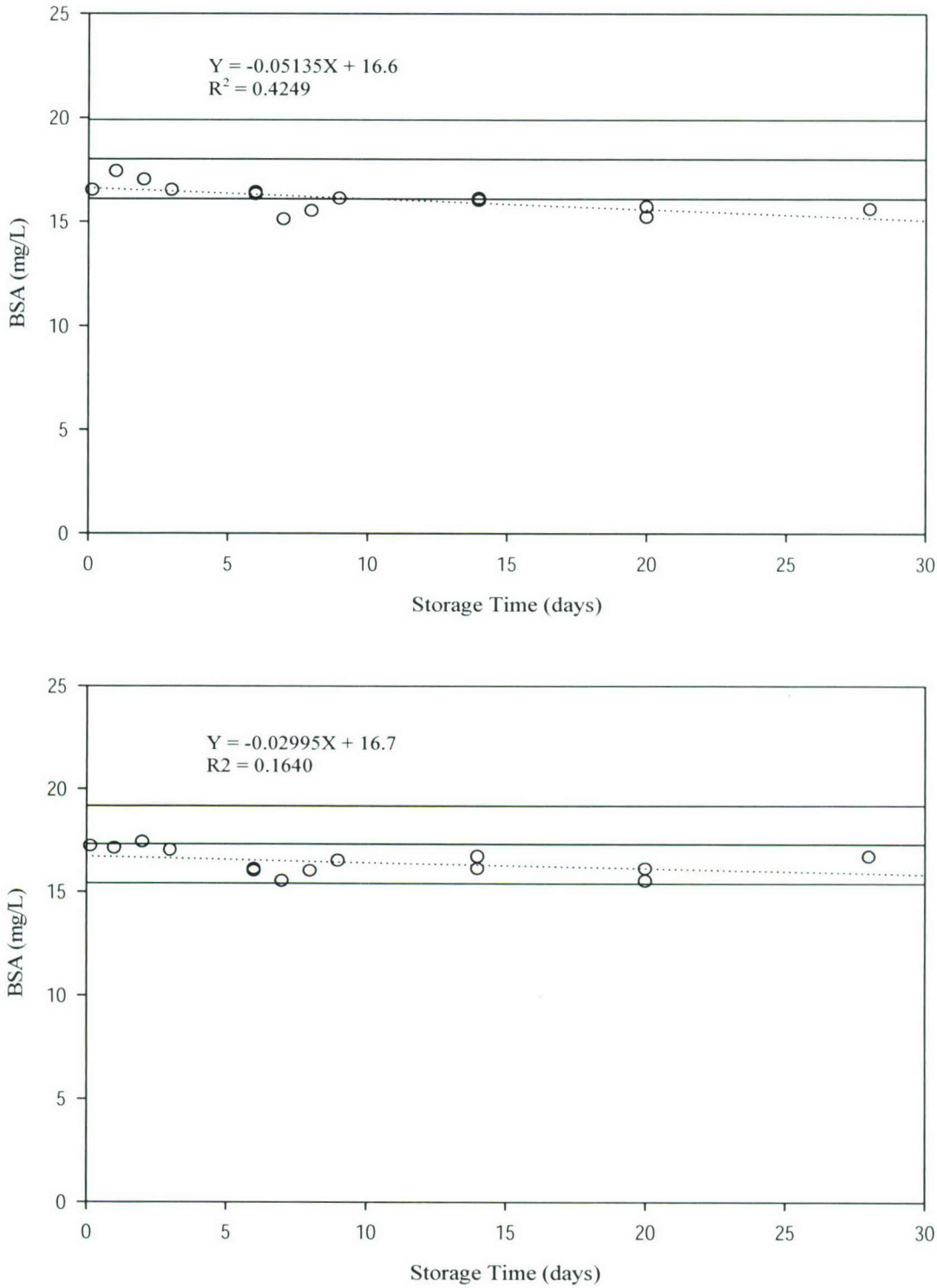

Figure 13. Stability of BSA Solution Stored at 40 °C. The upper panel is the unfiltered sample, and the bottom panel is the filtered sample. The dotted line is the linear regression line and the solid lines are the average, minimum and maximum BSA concentration at time zero. Each open circle represents the average of eight measurements on one sample, with two samples being analyzed on days 6, 14, and 20 and single samples analyzed at the other time points.

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